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Cloning and characterization of a cDNA encoding a cobalamin-independent methionine synthase from potato (*Solanum tuberosum* L.)

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Abstract

A potato cDNA clone, *StMS*1, that encodes a methionine synthase was isolated. This protein was identified on the basis of both structural and functional evidence. The predicted sequence of the protein encoded by *StMS*1 shows a high degree of similarity to methionine synthases from other organisms and the expression of *StMS*1 in bacterial mutant strains restored the mutant's ability to synthesize methionine. Genomic organization and expression analyses suggest that *StMS*1 is a low-copy gene and is differentially expressed in potato organs. *StMS*1 expression was found in all tissues, but at elevated levels in flowers, basal levels in sink and source leaves, roots and stolons, and low levels in stems and tubers. RNA expression data were confirmed by western blot analysis except that the protein content in leaves was less than expected from the RNA data. Western blot analysis of subcellular fractions revealed that the protein is located in the cytosol. However, the changing pattern of gene expression during the day/night period implied a light-dependent control of MS transcription normally seen for enzymes localized in plastids. The expression of MS was shown to be light-inducible with its highest expression at midday. These RNA data were not confirmed at the protein level since protein content levels remained constant over the whole day. Feeding experiments of detached leaves revealed that sucrose or sucrose-derived products are responsible for *StMS*1 induction. This induction can be blocked by treatment with DCMU during the light period. Western analysis revealed that the amount of *StMS*1 is not affected by either treatment. This experiment confirmed the presence of a day/night rhythm. Methionine synthase expression is regulated by photoassimilates but this seems not to detectably alter protein levels.

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; MS, methionine synthase

Introduction

Methionine (Met), an essential amino acid, belongs to the aspartate family of amino acids derived from the common precursor aspartate (Matthews, 1999). Plants and micro-organisms are able to synthesize Met while man and monogastric animals have to derive this amino acid from their diet. In plants Met serves as precursor for a variety of metabolic processes, including protein synthesis, as the prime methyl donor for a large number of biological methylations, polyamine synthesis (Tiburcio *et al.*, 1990), and ethylene synthesis (Kende, 1993). Since methionine synthase is also required for both the regeneration and the *de novo* biosynthesis of Met (Cossins, 1987; Anderson, 1990), it is the convergence point for two major biochemical

The nucleotide sequence data reported will appear in the EMBL database under the accession number AF082893.

domains in cellular metabolism: the Met biosynthetic pathway and the one-carbon cycle.

Methionine synthase catalyses the final reaction of the Met biosynthetic pathway. The first step of the pathway is catalysed by the enzyme cystathionine γ-synthase (CgS) to form cystathionine from the substrates cysteine and *O*-phosphohomoserine. It is important to note that *O*-phosphohomoserine is also the immediate precursor of threonine so that methionine synthesis and threonine synthesis compete for a common substrate. The reaction catalysed by CgS is followed by the conversion of cystathionine to homocysteine by the enzyme cystathionine β-lyase. In the last step, a methyl group is transferred in plants from N^5-methyl-tetrahydrofolic acid to homocysteine by a vitamin-B_{12}-independent methionine synthase (EC 2.1.1.14) to yield Met (Giovanelli *et al.*, 1980).

In *Escherichia coli*, two types of methionine synthases have been described. A cobalamin-dependent methionine synthase (metH, EC 2.1.1.13) is encoded by the *met*H gene and contains a cobalamin prosthetic group that is required for activity. Another cobalamin-independent methionine synthase (metE, EC 2.1.1.14) is encoded by the *Met*E gene and has no known requirement for a vitamin B_{12}-derived prosthetic group. Both enzymes catalyse essentially the same overall reaction, in which a methyl group from 5-methyltetrahydrofolate is transferred to the thiol group of homocysteine to generate Met and tetrahydrofolate (Gonzáles *et al.*, 1992; Golding and Matthews, 1997; Brand *et al.* 1998). The presence of *met*E and *met*H indicates the ability of an organism to synthesize vitamin B_{12} or to obtain it from its environment.

Methionine synthases have been investigated biochemically and several genes have been cloned recently. Most data have been obtained with partially purified enzymes and recombinant protein (Cossins, 1980; Madison, 1990; Eichel *et al.*, 1995; Eckermann *et al.*, 2000). Apparently, the enzymes are of a cobalamin-independent type (metE type) that use methyltetrahydrofolate polyglutamates as methyl donors but in a few cases also accept monoglutamate as substrate. The occurrence of subcellular methionine synthase activity compartmentalization is still a matter of controversy (Wallsgrove *et al.*, 1983; Ravanel *et al.*, 1998). Enzyme activity has been assigned to the cytosol but has also been detected in mitochondria (Clandinin and Cossins, 1974) and plastids (Shah and Cossins, 1970). However, most data indicate that the MS protein is predominantly cytosolically localized (Eichel *et al.*, 1995; Gakière *et al.*, 1999; this paper).

Genes encoding methionine synthases have been isolated from different organisms. For higher plants, full-length cDNAs were reported for *Catharanthus roseus* (Eichel *et al.*, 1995; X83499), *Chlamydomonas reinhardtii* (Kurvavi *et al.*, 1995; U36197), *Chlamydomonas moewusii* (U77388) and *Coleus blumei* (Petersen *et al.*, 1995; Z49150) or published in the database for *Arabidopsis thaliana* (Gakière *et al.*, 1999; U97200) and *Mesembryanthemum crystallinum* (U84889).

The cloning and characterization of a cobalamin-independent methionine synthase from potato is described here. Since a number of questions such as tissue-specific localization, copy number, the detailed role of the enzyme and its reaction mechanisms remain open, the isolated potato cDNA was characterized in order to contribute further data concerning the regulation of Met synthesis in higher plants.

Materials and methods

Strains and plants

Escherichia coli XL-1-blue (Stratagene) was cultured using standard techniques (Sambrook *et al.*, 1989). For complementation, two Met auxotrophic strains, GS243 (*phe*A905 *thi* Δ*lacU169* and *ara*D*129 rps*L Δ*met*E::Mu) and GS472 (*trp*R *lac*Z *met*E70 *met*H) (Urbanowski *et al.*, 1987), were cultured on M9 medium supplemented with amino acids, thiamine and IPTG and, for a positive control, methionine (40 mg/l) as described by Sambrook *et al.* (1989). The solid medium contained 1.5% w/v agarose (Gibco). Plates were incubated at 37 °C.

Potato plants (cv. Désirée, Saatzucht Fritz Lange, Bad Schwartau, Germany) were cultivated under a 16 h light/8 h dark regime in soil in the greenhouse. Plants used for light induction experiments were kept in the greenhouse under normal day/night conditions with an average temperature of 22 °C. Leaves used for feeding experiments were cut while submerged and then incubated in either tap water or tap water plus 250 mM sucrose and 100 μM DCMU, respectively, for 24 h. Tissue harvested for analyses was frozen immediately in liquid nitrogen and stored at −80 °C.

DNA and RNA manipulation

DNA manipulations were carried out essentially as described by Sambrook *et al.* (1989). The cDNA insert was subcloned into pBluescript SK^- and both

strands were sequenced with T7 polymerase (Pharmacia) using standard and synthetic oligonucleotides (TibMolbiol, Berlin, Germany). Sequence data were analysed using the programs of the Wisconsin GCG package (Devereux *et al.*, 1984). High-molecular-weight DNA was extracted from potato plant leaves as described by Rogers and Bendich (1985). Genomic DNA was digested with various restriction enzymes and was separated electrophoretically in 0.8% agarose gels using TBE buffer. The fragments were transferred onto a nylon membrane (Porablot, Macherey-Nagel, Germany) as described by the manufacturer's instructions. Total RNA was isolated according to the protocol of Logemann *et al.* (1987) from potato leafs, stem, roots, tubers and flowers of 8-week old potato plants. The isolated RNA was fractionated on a 1.3% agarose/formaldehyde gel and blotted onto the same type of filters used for Southern hybridization. Blots were probed with randomly [^{32}P]dCTP-labelled *StMS*1 cDNA using the Ready Prime kit (Amersham) in hybridization buffer containing 250 mM sodium phosphate buffer pH 7.2, 250 mM sodium chloride, 1 mM EDTA, 7% SDS, 6.6% PEG, 10 mg/ml SS-DNA and 20% formamide at 45 °C. The last wash was performed for 20 min in 0.1× SSC, 0.1% SDS.

For the complementation of the *E. coli* mutant strains GS243 and GS472, potato *StMS*1 was cloned into the expression vector pKK 388-1 (Clontech, USA) using the following procedure. A DNA fragment encoding the mature protein of MS from potato was amplified by polymerase chain reaction generating an *Eco*RI site at the 5′ end using a synthetic oligo (5′-GAGAGAATTCATGGCATCTCACGTTGTT-3′) and a T3 primer at the C-terminal end. The PCR product was ligated into *Eco*RI and *Asp*718 restriction sites of the expression vector.

Protein extraction, SDS-PAGE, western blot analysis, and antibody preparation

Total protein was extracted from leaf discs (ca. 250 mg) of 8-week old plants, frozen in liquid nitrogen, and stored at −80 °C. Leaf tissue was homogenized at 4 °C with 500 µl of buffer containing 1 mM EDTA, 20% v/v glycerol, 10 mM 2-mercaptoethanol and 5 mM thiourea in 100 mM potassium phosphate pH 8, followed by centrifugation at 4 °C (1500 × *g* for 20 min). Protein concentration was measured by the method of Bradford (1976) with BioRad protein assay reagent and bovine serum albumin as a standard. Extracts were stored at −80 °C until assayed.

For western blot analysis, protein samples were mixed with equal volumes of sample buffer consisting of 250 mM Tris-HCl pH 6.8, 30% w/v glycerol, 0.3 M DTT, 6% SDS, and 0.01% w/v bromophenol blue and were then denatured at 95 °C for 10 min. Protein samples were analysed by SDS-PAGE (Laemmli, 1970) with mini gels (BioRad, München, Germany) containing 10% of a 30% acrylamide/bisacrylamide stock solution (BioRad) in the separation gel. After electrophoresis in 25 mM Tris-base pH 8.3, 250 mM glycine and 0.1% SDS, the gels were equilibrated in transfer buffer (48 mM Tris-base pH 8.3, 39 mM glycine, 0.0375% w/v SDS, 20% v/v methanol) for 10 min followed by transfer of the proteins onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) with a semi-dry electroblotting apparatus (BioRad). Recombinant potato methionine synthase protein was synthesized as a partial 40 kDa (containing the C-terminal part of MS) 6× His tag fusion protein from the pQE-30 vector (Qiagen, Hilden, Germany) expressed in *E. coli*. Rabbits were directly immunized with a polyacrylamide gel slice containing the antigen (Pineda Antibody Service, Berlin, Germany). This polyclonal antibody was capable of detecting the MS protein in 5 µg total leaf protein using either colorimetric detection (with a biotinylated secondary antibody and streptavidin-conjugated alkaline phosphatase) or Amersham's ECL western blotting analysis system (with a conjugated horseradish peroxidase-labelled secondary antibody). The ECL immunodetection procedure was done following the protocol described in the user's manual. By mixing *in vitro* transcribed and translated radioactive ^{35}S-labelled MS protein with plant crude extract, we were able to show the specificity of the primary antibody by comparing autoradiographs with western blots. The mature MS protein was labelled by an *in vitro* transcription, translation reaction combined in a TNT-coupled reticulocyte lysate kit from Promega (Germany). The reaction was performed as described in the manufacturer's instructions.

Enzyme assays

If not described otherwise, enzymes were assayed spectrophotometrically at 340 nm, by measuring the coupled oxidation/reduction of NADH or NADPH in 1 ml reaction volumes.

UDP-glucose pyrophosphorylase (UPGase) activity was assayed in 0.1 M Tris-HCl pH 8.0, 2 mM $MgCl_2$, 0.25 mM NADP, 2 mM NaF, 20 μM glucose-1,6-bisphosphate, 2 mM UDPgluc, 3 U/ml phosphoglucomutase from yeast, and 1 U/ml glucose-6-phosphate dehydrogenase from yeast, at 25 °C. The reaction was started with 2 mM $Na_2P_2O_7$ (final concentration).

ADP-glucose pyrophosphorylase (AGPase) activity was measured in a reaction assay containing 80 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM ADP-glucose, 0.6 mM NAD, 10 μM glucose-1,6-bisphosphate, 3 mM DTT, 0.02% BSA, 1 U phosphoglucomutase, 2.5 U NAD-linked glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) and protein extract at 30 °C. The reaction was initiated by the addition of sodium pyrophosphate (2 mM final concentration).

Results

Isolation and characterization of the potato methionine synthase StMS*1 gene*

A. Saccharomyces cerevisiae mutant strain, MB192, that is deficient for *pho*84 was used to isolate genes encoding phosphate transporters from *Solanum tuberosum* (Leggewie *et al.*, 1997). One of the sequenced cDNA inserts, designated *StMS*1, encodes methionine synthase. The cDNA has a length of 2644 bp with an open reading frame of 2295 bp, a putative polyadenylation signal AATAAA (Joshi, 1987), and a short poly(A) tail. *StMS*1 encodes a peptide of 765 amino acids with a molecular mass of 84.7 kDa. Sequence comparison of the predicted amino acid sequence of this putative potato MS with the deduced sequences from *Arabidopsis thaliana*, *Catharanthus roseus*, *Clamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Coleus blumei*, *Mesembryanthemum crystallinum*, and *Escherichia coli* showed highly conserved sequence stretches (data not shown) and identities ranging between 38.7% and 90.3% (Table 1). The amino acid residues from the start methionine do not show features typical of a transit peptide such as a high serine content and positively charged or hydrophobic side chains (Keegstra and Olsen, 1989; Gavel and van Heijne, 1990). A carboxyl-terminal motif, WVNPDCGLKTR, is also present that seems to be strictly conserved in cobalamin-independent methionine synthases from plants, micro-organisms and algae. It is assumed that this motif is part of the active site of the enzyme as studies with the *E. coli* enzyme demonstrated. The cysteine residue in this motif functions as a zinc ligand suggesting that this metal is required for catalysis (Gonzáles *et al.*, 1996; Brand *et al.*, 1988).

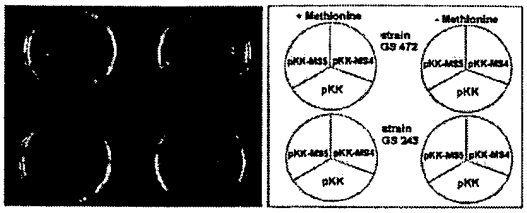

Figure 1. Complementation of the *E. coli* methionine synthase-deficient strains GS243 and GS472 by potato *STMS*1. Transformed cells containing the expression vector pKK 388-1 alone as control or including the cDNA coding for potato methionine synthase were plated on minimal media supplemented and not supplemented with methionine.

Functional complementation of bacterial methionine synthase mutants

To confirm that the *StMS*1 clone encodes a functional methionine synthase gene, its ability to complement methionine synthase-negative mutant strains of *E. coli* was investigated. The bacterial strains GS243 and GS472 are both defective in the *met*E gene and GS472 also has a defective *met*H gene (Umbarger, 1978). These strains were used in transformation studies. The entire *StMS*1 open reading frame was cloned into the pKK 388-1 vector to form the construct pKK-StMS1. The correct cloning was confirmed by construct sequencing. Both bacterial mutant strains were transformed with pKK-StMS1 and as control the empty pKK 388-1 vector. Transformant growth was assayed on minimal medium without cobalamin in the presence or absence of methionine (Figure 1). The expression of *StMS*1 permitted the growth of both bacterial strains on minimal medium without methionine while the insertless, vector-transformed mutant is unable to grow.

Southern analysis of the StMS*1 gene*

As an indicator of how central the metabolic function of MS is in plant metabolism, the potato genome copy number was tested. Southern analysis was performed with genomic DNA digested with *Eco*RI, *Hin*dIII, *Pst*I, *Eco*RV, *Asp*718, *Bam*HI and *Xba*I, with the full-length *StMS*1 cDNA as a probe (Figure 2). The hybridization result suggests that *StMS*1 is a

Table 1. Amino acid sequence identity (%) between different cobalamin-independent methionine synthases from plants, algae and the *E. coli* protein

	St	Cro	Cb	Mc	At	Ec	Cr	Cm
St		**90.3**	**89.8**	**89.7**	**89.4**	**49.9**	**43.4**	**38.7**
Cro	*94.5*		**89.3**	**87.7**	**88.2**	**49.7**	**43.2**	**39.8**
Cb	*94.7*	*93.8*		**86.6**	**86.4**	**50.0**	**42.1**	**40.2**
Mc	*93.1*	*92.8*	*92.0*		**89.7**	**49.7**	**42.9**	**38.6**
At	*94.0*	*92.9*	*92.8*	*94.6*		**49.5**	**43.4**	**38.8**
Ec	*67.1*	*66.8*	*66.6*	*66.8*	*66.9*		**44.3**	**38.8**
Cr	*61.9*	*62.0*	*61.3*	*62.3*	*62.4*	*63.5*		**36.1**
Cm	*57.0*	*57.7*	*57.2*	*56.9*	*57.2*	*59.7*	*56.4*	

Amino acid sequence identity is given in bold, similarity in italics. Percentage of identity and similarity were calculated with the help of the program Bestfit of the Wisconsin Genetics Group (GCG Package, Version 8.1; Devereux *et al.*, 1984). *Solanum tuberosum* (ST; this paper; AF082893), *Catharanthus roseus* (Cro; Eichel, 1995; X83499), *Coleus blumei* (Cb; Petersen 1995; Z49150), *Mesembryanthemum crystallinum* (Mc; U84889), *Arabidopsis thaliana* (At; Gakière *et al.*, 1999; U97200), *Escherichia coli* (Ec; M87625), *Chlamydomonas reinhardtii* (Cr; Kurvavi, 1995; U36197), and *Chlamydomonas moewusii* (Cm; U77388).

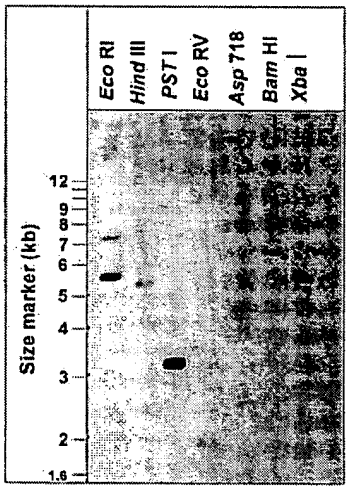

Figure 2. Genome organization of *STMS1*. Southern analysis of the *STMS1* genomic region. DNA was extracted from potato plants and 20 μg were digested with the indicated restriction enzymes. The probe used was the *StMS1* cDNA. Hybridization and washes were performed under low- and high-stringency conditions. The sizes of the marker, in kb, are indicated on the left.

low-copy gene. Even hybridizations and washes at low-stringency conditions revealed no differences in hybridization patterns (data not shown). Despite its diverse involvement in plant metabolism hybridization results indicate the presence of only one or a few copies of MS within the genome and fail to indicate the presence of additional, distantly related isoforms.

Expression of the potato STMS*1 gene and the intracellular localization of its encoded protein*

The expression of the *StMS*1 gene was analysed by RNA blot hybridization of RNA extracted from different potato organs. By using total RNA preparations and *StMS*1 cDNA as a probe a transcript of about 2.7 kb was detected in all organs investigated but at different steady-state transcript levels (Figure 3A). The highest amount of transcript was detected in flowers. Less expression was observed in sink and source leaves, roots, and stolon, but RNA levels were markedly low in stems and tubers. Western blot analysis of total MS protein of different potato tissues confirmed the expression data. Only in sink and source leaf tissues was the MS protein amount less than expected from the RNA data (Figure 3B). In western blot analysis, primary antibody reacted with a second,

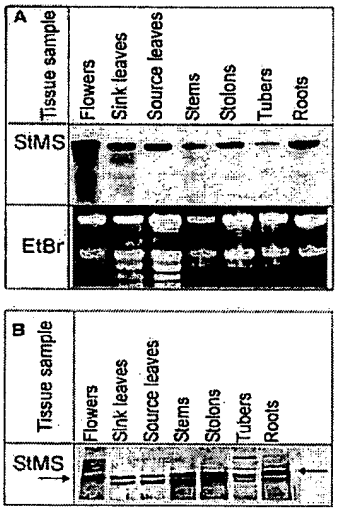

Figure 3. Expression of *STMS1* in different organs of potato. A. Total RNA blot hybridizations of 30 μg extracted from flowers, sink and source leaves, stem, stolon tubers, and roots of 8-week old plants were performed at 42 °C under high stringency. The probe was a 1.3 kb DNA *Bam*HI/*Sac*I fragment of the *StMS1* cDNA. Ethidium bromide-stained RNA is shown as a loading control. B. Total protein of flowers, sink and source leaves, stem, stolon tubers and roots was extracted and analysed by protein blot experiment with polyclonal antisera raised against MS. MS was detected in 5 μg total leaf protein.

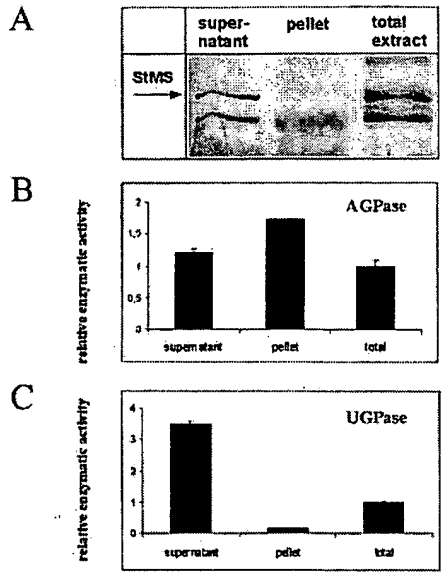

Figure 4. Localization of MS in subcellular fractions of potato leaf extracts. Total protein of extracts were analysed in western blot experiments with 5 μg protein. Specific activity of supernatant and organelle fractions are given in relative values related to the activities in crude extracts. ADP-glucose pyrophosphorylase (AGPase) and UDP-glucose pyrophosphorylase (UPGase) are measured as plastidial and cytosolic marker enzymes, respectively.

smaller protein in extracts from all tissues tested with the exception of flowers. By spiking the protein extract with radiolabelled MS it was established that the upper protein is the full-length MS protein, but these data cannot rule out the possibility that the smaller protein is also a MS (data not shown).

To investigate the subcellular localization of the MS protein, leaves were homogenized and fractionated by centrifugation to yield membrane (pellet) and cytosolic (supernatant) fractions. Fractions and crude homogenates were assayed for MS protein in western blot analysis (Figure 4A). The quality of the fractions was assessed by measurement of selected subcellular marker enzyme activities. UDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase were used as marker enzymes for the cytosolic and chloroplastic (membrane) fractions, respectively (Figure 4B and C). As shown in Figure 4B, the membrane fraction was free of cytosolic marker activity, while the supernatant was contaminated by chloroplast-derived enzymatic activity. Since the MS protein was detectable in only the cytosolic fraction, it can be clearly deduced that it is cytosolic.

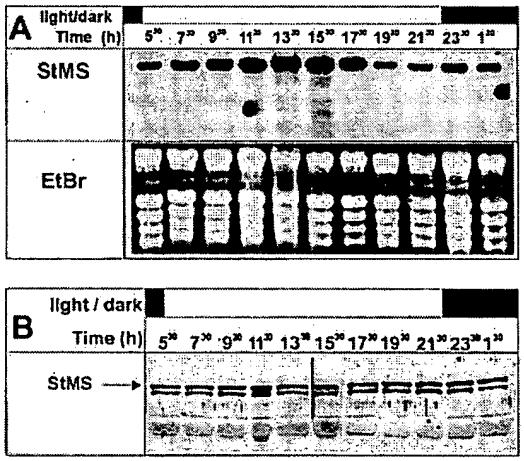

Figure 5. Time course expression of methionine synthase during light and darkness. Samples were taken every 2 h. A. RNA blot analysis of total RNA from *S. tuberosum* with *STMS1* as a probe. Total RNA (40 μg) was separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane and hybridized to the full-size cDNA. Ethidium bromide-stained RNA is shown as a loading control. B. Comparison of methionine synthase protein amounts in western blot analysis with 5 μg total protein.

To investigate whether MS expression is modulated by external and internal factors, the effect of light was tested. Potato plants were kept in the greenhouse and leaf samples were collected every two hours to prepare total RNA. Northern blot analysis revealed that MS showed a light-dependent modulation of expression with the highest amounts of mRNA being detected at midday (Figure 5A). At the end of the illumination period, the transcript level of MS declines reaching a basic transcription level that remains constant in darkness.

Protein levels of MS were analysed in parallel in protein blots of leaf protein extracts (Figure 5B). Intriguingly, the protein amount exhibited no significant change in levels during the day. This indicates that although the MS transcript level is increased MS protein level is not influenced by the accumulation of MS transcript.

Methionine synthase is transcriptionally regulated by photoassimilates but is unchanged on protein level

Synthesis of amino acids requires both carbon and nitrogen. To analyse whether photoassimilates are responsible for the modulation of *StMS*1 transcription in vegetative tissue feeding experiments of detached leaves were performed in light and in darkness. To evaluate whether the availability of carbon sources like sucrose exerts an effect on the expression of *StMS*1 detached potato leaves were incubated with water (Figure 6A, control), sucrose (Figure 6A, sucrose), and DCMU (Figure 6A, DCMU) for 24 h in light (open panel) or darkness (black panel). Total RNA was extracted and subjected to northern blot analysis using *StMS*1 cDNA as a probe. As shown previously in Figure 5A, the *StMS*1 transcript level is down-regulated in darkness, but, in the same light conditions when the water is supplemented with sucrose, a significant increase of *StMS*1 expression results. To analyse whether light *per se* or photoassimilates are responsible for the transcriptional regulation DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea) was used to block photosynthesis. The transcription of *StMS*1 is therefore down-regulated independently of illumination status. ADP-glucose pyrophosphorylase (AGPase), the starting point for plant starch biosynthesis, was used as a control for *StMS*1 expression. *StMS*1 follows AGPase S expression (Figure 6A), which is regulated by light sucrose, and/or photoassimilates (Müller-Röber *et al.*, 1990). However, in this study, western blot analysis revealed that the protein content

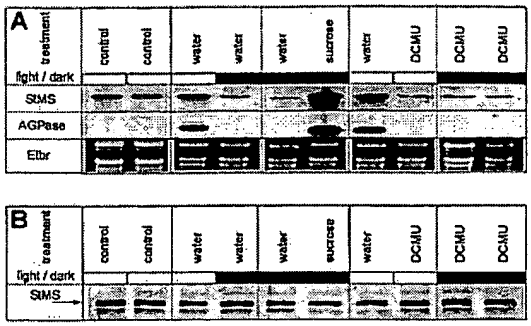

Figure 6. Expression of MS in light and darkness in the presence of sucrose and DCMU, respectively. Total RNA and protein were extracted from detached potato leaves directly (control) or after incubation in light or darkness for 24 h in water and water supplemented with sucrose and DCMU, respectively. A. Total RNA (40 μg each) was hybridized to cDNA inserts of MS and AGPase S as control. Ethidium bromide-stained RNA is shown as a loading control. B. Comparison of methionine synthase protein amounts in western blot analysis with 5 μg total protein.

of StMS1 is not influenced by its transcriptional regulation (Figure 6B). Taken together these data suggest that *STMS*1 expression is regulated by sucrose or photoassimilates, reaching its highest transcript levels at midday, but that this increase in transcription has no measurable effect on protein content.

Discussion

The metabolic role of methionine synthase in the context of plant growth and development remains to be fully clarified. In this report, the isolation of the potato methionine synthase cDNA (*STMS*1) and its initial characterization with respect to gene expression and protein level are described. The *STMS*1 cDNA was identified based upon two lines of evidence. First, the sequence has high homology to known methionine synthase sequences from plants (89.4–90.3%) and *E. coli* (metE: 49.9%), and second, bacterial mutant strains GS243 and GS472, devoid of MS activity due to defects in the corresponding genes *met*E and *met*E/*met*H, respectively, could be functionally complemented with *StMS*1.

The intracellular localization of methionine synthase in leaves is controversial (Ravanel *et al.*, 1998). Wallsgrove *et al.* (1983) fractionated pea and barley leaf protoplasts and determined that only 4% of the methionine synthase in crude lysates was associated with the chloroplasts, the bulk (84%) was present in the cytosolic fraction, and 12% in the mitochondria.

An explanation could be that Met derived from aspartate is synthesized by a plastidic enzyme, whereas in the cytosol the methylation of homocysteine is catalysed by a cytosolic enzyme as a cycling system for the C1 metabolism intermediate, homocysteine. However, in this paper we used western blot analysis to show that the STMS1 protein is present only in the supernatant (i.e. cytosolic fraction) of potato leaf extracts. A cytosolic localization is further corroborated by the fact that the deduced potato sequence appears to lack a transit peptide. Furthermore, these data are in accord with the western blot analysis of methionine synthases from *C. roseus* and *A. thaliana* (Eichel *et al.*, 1995; Gakière *et al.*, 1999) and indicate that an exclusively cytosolic localization of plant methionine synthases is likely.

In most of the potato tissues examined, western blot analysis revealed two bands. Experiments with crude protein extracts mixed with *in vitro* labelled MS protein verified that the higher-molecular-mass band is the full-length MS protein. Such a double band has been observed for a number of different plant species belonging to Apiacea and Moracea families reacting with polyclonal antibodies against the *C. roseus* protein (Eichel *et al.*, 1995; Eckermann *et al.*, 2000). The appearance of a smaller protein in extracts from potato belonging to the solanaceous family leads to the assumption that the presence of such a putative shortened MS is a widespread phenomenon. In parsley, activity measurements suggested that the smaller protein is also a cobalamin-independent MS, with properties comparable to the entire MS protein (Eckermann *et al.*, 2000). The identity and function of the smaller, possibly processed protein in potato remains unclear and should be established in future.

In potato, *STMS*1 hybridizes to only one 2.7 kb transcript when used as a probe for northern blot analysis. This is supported by genomic DNA blot analysis. The low complexity of the pattern of genomic fragments suggests that the *StMS*1 gene represents a low-copy gene in the potato genome using both stringent and non-stringent hybridization and washing conditions, respectively (data not shown for non-stringent conditions). Results of the northern experiments show the expression of the potato *STMS*1 gene to be differentially regulated in a tissue-specific manner. Transcription of *STMS*1 was found to be more prominent in flowers than in sink and source leaves, stolon, and roots. Lowest transcript amounts were determined in stem and tubers. This result correlates with protein analysis of various potato tissues. Only in sink and source leaves was the protein amount less than expected from the RNA data. The lowest protein content was seen in tuber extracts. A similar expression pattern has been observed for cystathionine γ-synthase from potato (Riedel *et al.*, 1999). From these data it has been concluded that, though at different levels, methionine biosynthesis occurs in all plant tissues, not only in source organs, although the rate may differ in different organs. The data support the recent findings of Nam *et al.* (1999) and Zegzouti *et al.* (1999), which showed that CgS in strawberry and MS in tomato fruits, respectively, are highly expressed during fruit formation. This might indicate that there is a higher demand for Met during flowering and fruit formation and might explain the increase in MS RNA and protein, again supporting the local formation of Met. It is therefore reasonable to speculate that amino acid biosynthesis, especially Met biosynthesis, occurs in sink tissues like fruits and flowers in order to attain required intracellular amino acid concentrations. Furthermore, methionine synthase might be co-regulated with CgS to meet the demand for Met.

Several amino acid biosynthetic pathway genes follow a day-and-night rhythm in their expression (Raines *et al.*, 1991; Willingham *et al.*, 1994). By investigating the effects of environmental factors such as light on *STMS*1 expression, it was demonstrated that *STMS*1 expression occurs in a light-dependent manner, as does the expression of several nuclear-encoded plastid-localized enzymes (Gallagher *et al.*, 1985). The present investigations show that methionine synthase is transcribed at a basal level at night in potato leaves and that the transcript concentration rises when leaves are illuminated, reaching the highest expression at noon. The expression declines in the afternoon, reaching a steady-state level after the light is switched off. The cell's metabolic state (i.e. the availability of photosynthesis products) might be responsible for this transcriptional modulation as has been shown for plant genes encoding enzymes for nitrogen metabolism and for sulfur assimilation (Small and Gray, 1984; Edwards and Coruzzi, 1989; Melzer *et al.*, 1999; Riedel *et al.*, 1999).

Carbon partitioning between carbohydrates and amino acids is subject to a complex metabolic regulation system triggered by light, photosynthesis-related metabolites, and nitrogenous compounds (Champigny and Foyer, 1992; Staswick, 1994; Lam *et al.*, 1994, 1995; Galili, 1995). It has been found that at high sugar but limiting nitrogen levels, carbohydrates accumulate preferentially, whereas under conditions of

high carbon and high nitrogen levels, a larger proportion of the carbon is shifted towards amino acid and protein production. In the present study, the physiological reason for the gene induction was investigated by feeding experiments using detached leaves. The light-dependent but late-peaking expression of *STMS*1 suggests that photoassimilates accumulating slowly during the illumination period might be responsible for gene induction. To test this hypothesis the expression of *STMS*1 was followed in detached leaves in light and darkness and in the presence of sucrose and/or DCMU in northern and western blot experiments. These experiments revealed that *StMS*1 expression is regulated at the level of transcription. Data obtained show that the amount of carbohydrate produced by the photosynthetic activity of the leaf is sufficient to induce accumulation of *StMS*1 RNA. That photosynthesis is required for this effect and not light per se is demonstrated by the failure to induce *StMS*1 accumulation upon incubation with DCMU, a specific inhibitor of photosynthesis, in light and, furthermore, by the fact that exogenously added sucrose induces *StMS*1 mRNA efficiently in the dark. The expression of *StMS*1 follows the pattern observed for ADP-glucose pyrophosphorylase S from potato, which was used as a control (Müller-Röber *et al.*, 1990). These data are compatible with the assumption that increased levels of photoassimilates trigger this modulation of *STMS*1 expression.

Results of the concomitant protein analysis, however, suggest that the protein level is regulated independently from the expression level, since the protein level remained constant over the whole investigated time period. Likewise, the feeding experiments with detached leaves confirmed that *STMS1* expression is regulated at the level of transcription but not at the level of protein. Similar results have been reported for methionine synthase, SAM synthetase, and *S*-adenosyl-homocysteine hydrolase from *C. roseus* (Eichel *et al.*, 1995; Schröder *et al.*, 1997). It has been demonstrated in *C. roseus* suspension cells that the expression of MS is regulated with respect to nutritional status, growth phase, and elicitor treatment but that, as in our results, no significant change of the protein level is apparent (Eichel *et al.*, 1995; Eckermann *et al.*, 2000). One possible mechanism for such post-transcriptional regulation is the control of protein stability by its own N-terminal region. In this way, for example, nitrate reductase (NR) is regulated in a light-dependent manner (Vincentz and Caboche, 1991; Nussaume *et al.*, 1995). Conserved residues of NR's N-terminal amino acid sequence, DYSSSEDDDDDDEKNEY, have proved to be responsible for its stability. Although such a motif could not be identified within MS, further database searches revealed a region with low homology to a PEST sequence (HITPEDGFSYASFEA) present in several other enzymes, including two *S*-adenosylmethionine decarboxylase genes (Rogers *et al.*, 1986; Lee *et al.*, 1997). PEST sequences have been shown to control protein degradation in eukaryotes (Rechsteiner and Rogers, 1996) and are postulated to be signals triggering rapid proteolysis. PEST sequences consist of residues rich in proline (P), glutamic acid (E), serine (S), and threonine (T), and have stretches of uninterrupted positively charged residues. The sequence was identified by the PEST-FIND program developed by Rechsteiner and Rogers (1996; http://www.expasy.ch/tools/#pattern). Despite the fact that MS's PEST-like sequence, ELGPDVNFSYASHKA, is conserved in other MS protein sequences from *Catharanthus roseus*, *Arabidopsis thaliana* and *Mesembryanthemum crystallinum*, the low similarity of this motif to known PEST sequences makes it unlikely to be used as such a motif. If it is true that parsley's smaller protein band has similar MS properties to the entire protein, a proteolytic mechanism for the MS protein can be assumed. The true identity and catalytic activity of this lower-molecular-weight protein and its role for Met synthesis and homocysteine recycling should be examined in the future.

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